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THERMAL AND HORMONAL FEMINIZATION OF ALL MALE YY NILE TILAPIA, *OREOCHROMIS NILOTICUS* L.

Ismihan Karayücel[1]*, David Penman[2], Sedat Karayücel[1] and Brendan McAndrew[2]

[1] Su Ürünleri Fakültesi, Ondokuzmayis Üniversitesi, 57000 Sinop, Turkey

[2]Institute of Aquaculture, University of Stirling, Scotland, FK9 4LA, UK

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Abstract

Hormonal and thermal sex reversal of YY male *Oreochromis niloticus* were compared. While similar percentages of females (34% and 32%, respectively) were produced, a significantly higher percent of intersex individuals (18.5%) was produced in the heat (36ºC) treatment than in the group treated with diethylstilbestrol (DES; 1.6%). The heat-treated groups had 62.6% survival, compared to 97.0% and 97.3% in the control and DES-treated groups, respectively. Our results demonstrate that a high temperature treatment can be an alternative to hormonal sex-reversal treatments for YY male *O. niloticus.* Although the low survival and high occurrence of intersex individuals may limit its use, it can be used to produce large numbers of YY male broodstock in countries where hormone use is illegal and/or consumer reaction to hormonally-treated fish is negative.

Introduction

Studies have shown that the phenotypic sex in fish can be altered by environmental factors such as temperature (Conover and Kynard, 1981), pH (Beamish, 1993; Römer and Beisenherz, 1996) and pollutants (Torblaa and Westman, 1980). The most commonly identified environmental variable inducing sex-change is temperature (Bull, 1983) which is known to be capable of overriding the genotypic sex determination in a small but increasing number of teleost fishes (see reviews by Strüssmann and Patiño, 1995; Baroiller and D'Cotta, 2001; Baroiller and Guiguen, 2001).

* Corresponding author. Tel.: +90-368-2876265, fax: +90-368-2876255,
E-mail: ismihank@hotmail.com

In fishes, thermal alteration of the sex ratio (Temperature Sex Determination, TSD) has been reported especially among the atherinids. The most comprehensive TSD study was on the Atlantic silverside, *Menidia menidia* (Conover and Kynard, 1981) in which low temperature favored the formation of females whereas high temperatures yielded more males. Recently, thermolability of sex determination has been established in various fish species (Strüssman and Patiño, 1995). Sullivan and Schultz (1986) found that in the livebearing teleost, *Poeciliopsis lucida*, one strain produced almost all male offspring at 30ºC and female-biased sex ratios at 24ºC while another strain produced a 1:1 sex ratio at both temperatures. These authors suggested that in *P. lucida* sex ratio is influenced by genetic and environmental factors. Strüssman et al. (1997) assessed the effect of temperature on the sex ratios in two other atherinid fish. In *Odontesthes bonariensis,* all-female progeny were produced at 17ºC whereas fish exposed to about 25ºC became male-biased. In contrast, sex ratios were balanced (1:1) in *Patagonina hatcheri* in the same temperature range, indicating strong genotypic control. In the channel catfish, *Ictalurus punctatus*, the sex ratio was significantly skewed towards females in fish treated at 34ºC whereas exposure to low and ambient temperatures did not affect the sex ratio (Patiño et al., 1996). Rearing at high temperatures of 25ºC and 30ºC resulted in significantly higher percentages of males than in fry reared at 20ºC in normal crosses of XX female with XY male loach. Male progeny were observed at 25º and 30ºC in gynogenetic all-female progenies of loach. Goto et al. (1999) reported that an all-male population of barfin flounder, *Verasper moseri*, was produced by rearing the fish at 18ºC and that the sex ratio was 1:1 when the fish were maintained at 14ºC. The sex ratio of marbled sole, *Limanda yokohame*, inclined towards male when the fish were reared at 15±2ºC for 115 days then gradually acclimated to 25ºC over 10 days (Goto et al., 2000). Thermolabile sex determination was also shown by Fujioko (2001) in honmoroko, *Gnathopogon caerulescens*. In four of six pairings, the proportion of females decreased significantly with an increase of temperature. The author suggested that sex determination in this species is close to female homogamety but influenced by temperature, genetic factors and genotype-temperature interactions.

The sex ratio of *Oreochromis aureus* was reported to be skewed towards females by raising the rearing temperature for fry of mixed sex (Yu and Lay, 1982, cited in Mair et al., 1990). On the contrary, Desprez and Mélard (1998) reported high male ratios at 34ºC. In one of seven experiments, 20% females were observed in putative all-male broods of *O. aureus* derived from neomales subjected to 32ºC (Mair et al., 1990). In *O. mossambicus*, two cold temperature experiments at 19ºC yielded a significant excess of males (89% and 78%) compared to the control (Mair et al., 1990).

The effect of temperature on sex ratio in *Oreochromis niloticus* was extensively studied by Baroiller et al. (1995 a,b). Treatment at 36ºC of *O. niloticus* fry of mixed sex resulted in an increased proportion of males (33-81%; Baroiller et al., 1995a). Baroiller et al. (1995b) reported that high temperatures (34-36ºC) significantly increased the proportion of males in mixed sex families of *O. niloticus* (69-91%) and red tilapia (Florida strain; 98.3%). All-female *O. niloticus,* derived from crossing XX neomales with normal XX females, were exposed to temperatures of ≥32ºC and the proportion of males increased from 0 to 91% (Baroiller et al., 1996). The author suggested that in *O. niloticus* sex is determined by genetic factors, temperature and the genotype-temperature interaction.

Abucay et al. (1999) exposed *O. niloticus* progeny of genotypes XX, XY and YY to a high temperature (36.7±5ºC). A greater percent of males was obtained in the putative all-female progeny whereas a lower percentage of males was obtained in the all-male (YY) progeny. The authors hypothesized that sex differentiation in YY males might be more labile than in normal males (XY).

Baras et al. (2001) found that the sex ratio skewed significantly towards males in four of nine mixed *O. niloticus* progenies reared at

35°C and in all nine progenies reared at 37ºC (78-100% males) whereas the sex ratio in the control groups never differed significantly from 1:1.

Kwon et al. (2002) showed that high temperature (36°C) overrides genetic sex determination by masculinizing XX and feminizing YY fish. Their results confirm that treatment with dietary aromatase inhibitor is mechanistically associated with Temperature Sex Determination (TSD) and suggest the bidirectional pattern of TSD in this species.

Manipulation of water temperature may provide an alternative to hormonal sex control in fish, overcoming negative consumer reaction to hormone treatment and chromosome manipulation (Strüssman and Patiño, 1995; Patiño, 1997). While hormone-sensitive periods of sex determination seem to require a similar timing and duration in *M. menidia* (Conover and Fleisher, 1986), *O. bonariensis* (Strüssman et al., 1997) and *O. niloticus* (Baroiller et al., 1995a,b), the appropriate combinations of temperature and duration must be determined (Strüssman and Patiño, 1995). The objectives of this study were to compare hormonal and thermal treatments and further elucidate the genetic bases of the sex determination mechanism in all-male YY progeny of *O. niloticus*.

Materials and Methods

Fish stock. The Egypt-Swansea-Philippine isolate (ESP) of the Egyptian strain of *O. niloticus* used in this study originally came from Lake Manzala, Egypt, and was transferred to the Institute of Aquaculture, University of Stirling, in the late 1970s. Some fish from this stock were given to the University of Wales, Swansea, in 1982 and from there elsewhere. The experimental YY males and YY neofemales used in the present study were transferred to the Institute of Aquaculture, Scotland, from the University of Wales in 1995.

Broodstock were reared in recirculating freshwater systems consisting of partitioned glass tanks of 120 x 44 x 30 cm, aerated by airstones coupled to a low-pressure blower unit. Lighting was adjusted to 12 light and 12 dark hours. The water temperature was maintained at 28+1°C. Fish were fed commercial trout feed (Trouw Aquaculture Nutrition, Russhive, UK) three times a day *ad libitum*.

Eggs were collected by applying gentle downward pressure to ovulating females above a clean sterile petri dish (100 mm diameter). The eggs were washed carefully with water, then divided into two batches. Milt was stripped from males in a similar manner using a glass capillary tube. Milt contaminated with water or urine was rejected. Two YY neofemales were crossed with two YY males to generate two single pair matings.

Eggs were fertilized *in vitro* by mixing the milt with the eggs and then adding 10-20 ml aquarium water. The fertilized eggs were left in the petri dish for 2-3 min for water hardening. Then they were washed and transferred to a recirculating incubation system for further development.

Experimental design. The progeny from each family was equally divided into nine batches after the yolk sac resorption stage (10 days after fertilization). Three batches of fry were treated by oral application of diethylstilbestrol (DES) for 11 days at 28±1°C (the optimum duration time reported by Mair and Santiago, 1994) in a static hormone treatment unit containing six plastic holding tanks (30 x 19 x 17 cm). Three batches constituted the control and were reared at 28±1ºC, also in the hormone treatment unit. The other three batches were exposed to 36ºC for 21 days (the optimum duration time reported by Abucay et al., 1999) in a glass aquarium (120 x 40 x 40 cm) containing four plastic holding tanks (30 x 19 x 17 cm). The system was filled with clean aerated tap water before the start of the experiment. The desired temperature was maintained using a 0-100ºC range thermostatic heater with a stirrer pump (Gallenkamp, Thermo stirrer 85, 220-240V, EEC) in the glass aquarium. The stirrer pump circulated the water around the plastic holding tanks.

Since an insufficient number of fry were obtained from the first spawning, fry were produced in a second spawning from the same parents and distributed among the treatements as above.

Two header tanks were used to fill the experimental units. A plastic header tank (60 x 44 x 41 cm) was used to top up the hormone treatment tanks and a thermostatic heater (Visi-Therm, UK) with a range of 18-32ºC maintained the water temperature at 28±1ºC. A glass header tank (62 x 30 x 30 cm) was used to top up the heat treatment unit. The water temperature was maintained at 32ºC with a thermostatic heater (Visi-Therm, UK) with a range of 18-32ºC and adjusted to 36ºC by adding boiled tap water before filling the plastic holding tanks in the heat treatment unit.

Aeration was provided to each plastic holding and header tank through an air pump. The water temperature in each plastic holding tank was checked three times a day.

DES-treated feed was prepared by the alcohol evaporation method to obtain a concentration of 1000 mg hormone per kg finely sieved food (Mair and Santiago, 1994). Feed for the control and heat-treated groups was prepared in a similar manner as the hormone-treated food but without the addition of the hormone. All groups were fed 3 times a day *ad libitum*. After 11 days of treatment with the DES-treated feed, these groups were fed the control food for 10 days.

On day 22, all groups were transferred to an early fry rearing system and reared for sexing by the gonad squash method as described by Guerrero and Shelton (1974). The aceto-carmine staining method (Guerrero and Shelton, 1974) was used to determine the sex of the juvenile fish (under 2 g, 1.5-2 months old).

Statistical analysis. Heterogeneity chi-square tests were used to compare the sex ratio and survival between replicates in the DES and heat-treated groups. Intersex individuals were not used in calculating the χ^2 for the sex ratios. Since no heterogeneity was detected between the experimental groups from each replicate, analyses of variance or the non-parametric Kruskal-Wallis test was applied on arcsine transformed percentages to test for differences in survival rate and sex ratio between the experimental groups according to the distribution of data.

Results

Results are shown in Table 1.

Discussion

The mean survival rate in the heat-treated groups was significantly lower than the mean survival rate in the controls and DES groups. A similar observation was reported by Abucay et al. (1999) and Kwon et al. (2002) in the same strain of *O. niloticus*. In their work, significantly lower mean survival rates (53.0% and 32.3%) were obtained in heat-treated (36ºC) groups of YY males than in the controls (90.67% and 96.4%). These results may indicate some loss of developmental stability in YY fish.

No female progeny were observed in any of the control groups. This is to be predicted from the sex-determination mechanism of a predominantly monofactorial genotypic system with male heterogamety and female homogamety (Penman et al., 1987; Mair et al., 1997), since the progeny came from crosses between YY neofemales and YY males. On the other hand, the existence of females (32.0±5.2%) in the heat-treated groups indicates that high temperature does change the sex of some YY male *O. niloticus.* This percentage is comparable to the mean female percentages of 49.2% (range 0-94.4%) and 32.3% (range 6.2-47.7%) obtained by Abucay et al. (1999) and Kwon et al. (2002) in thirteen and one families, respectively, of heat-treated YY male progenies from the same strain (ESP) of *O. niloticus*. On the other hand, Abucay et al. *(*1999) obtained lower female percentages (range 0-11.5%; mean 1.4%) in progenies from crosses of YY neofemales from ESP with YY males from Egypt-ICLARM strains of *O. niloticus* subjected to heat treatment at 36ºC. The authors attributed the differing temperature sensitivity of the pure-bred (ESP x ESP) and crossbred (ESP x Egypt-ICLARM) YY males to the level of inbreeding of the Egypt-Swansea strain which made the pure-bred progeny more sensitive to environmental extremes.

The mean percent of females (33.8±1.5%) in the DES-treated group was lower than the 78% females reported by Mair and Santiago

Table 1. Feminization of all-male (YY) *O. niloticus* by heat or hormonal treatment.

	Experiment I Control (replicate no.)			*Experiment II Control (replicate no.)*			*Experiment I DES treatment (replicate no.)*			*Experiment II DES treatment (replicate no.)*			*Experiment I heat treatment (replicate no.)*			*Experiment II heat treatment (replicate no.)*		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
No. fry (initial)	73	73	73	58	58	58	73	73	73	58	58	58	73	73	73	59	59	59
No. fry (final)	71	73	73	56	55	56	70	69	72	56	58	57	64	65	49	16	36	27
Survival[1] (%)	97.3	100	100	96.5	94.8	96.5	95.9	94.5	98.6	96.5	100	98.3	87.7	89	67.1	27.1	61	45.8
Sex ratio [2] (female:male: intersex)	0:30:0	0:30:0	0:58:0	0:43:0	0:22:0	0:33:0	19:41:0	18:40:0	16:25:0	13:24:1	13:27:2	18:29:1	23:21:9	20:20:4	11:22:7	2:1:2	4:12:4	2:10:1
Females (%)	0.0	0.0	0.0	0.0	0.0	0.0	31.6	31.0	39.0	34.2	30.9	37.5	43.4	45.4	27.5	40.0	20.0	15.4
Males (%)	100.0	100.0	100.0	100.0	100.0	100.0	68.4	69.0	61.0	63.2	64.3	60.4	39.6	45.5	55.0	20.0	60.0	76.9
Intersex (%)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.6	4.8	2.1	17.0	9.1	17.5	40.0	20.0	7.7

[1] at the end of the treatment, i.e., 11 days for the DES treatment and 21 days for the heat treatment.

[2] two months after the end of treatment.

	Control	*DES treatment*	*Heat treatment*
Mean survival for treatment±SE	97.5 ± 0.9^{b}	97.3 ± 0.8^{b}	62.9 ± 9.8^{a}
Mean percent females for treatment±SE	0.0 ± 0.0	33.8 ± 1.5^{a}	32.0 ± 5.2^{a}
Mean percent males for treatment±SE	100.0 ± 0.0^{b}	64.6 ± 1.6^{a}	49.5 ± 7.9^{a}
Mean percent intersex for treatment±SE	0.0 ± 0.0	1.6 ± 0.8^{a}	18.5 ± 2.5^{b}

SE = Standard error

Common superscripts in the same row are not significantly different.

(1994) using the same DES dose (1000 mg/kg) and a similar treatment period (10 days, starting after yolk sac resorption at 10 days after fertilization) in mixed sex progenies of *O. niloticus*. These results infer that feminization of the YY genotype by oral administration of DES may be more difficult to achieve than feminization of other genotypes using the same procedure. The hypothesis of differential feminization of XY and YY genotypes is supported by Abucay and Mair (1997, cited in Mair et al., 1997) and Karayücel (1999). The first authors investigated the sex ratios of feminized and nonfeminized males in DES-treated progeny of XY neofemale x YY male crosses and found evidence for this hypothesis in one of three families. The second author feminized the progeny from several crosses with DES to produce YY males and YY neofemales. A significantly lower percent ($p<0.05$) of females (range 0-51%; mean 24.0%) was obtained in crosses of a YY neofemale with a YY male than in crosses of XX female x YY male (47.7-100%; mean 77.7%), XY neofemale x XY male (50-96.9%; mean 75.3%) and XY neofemale x YY male (56.8-93.2; mean 83.6%).

Similar percentages of females (including intersex individuals) were produced by the DES treatment (35.4%) and the heat treatment (50.5%), suggesting that heat treatment at 36ºC for 21 days during sexual differentiation can be used as successfully as hormonal sex reversal for YY male *O. niloticus*. Strictly monosex females were not observed in this study.

Thermal treatment to affect sex ratios must begin before the onset of histological gonadal sex differentiation and at least partially overlap this critical period (Baroiller and Guiguen, 2001). Nakamura and Nagahama (1989) stated that histological differentiation of the gonads of *O. niloticus* occurs 23-26 days post-hatch at 25ºC. The duration of the temperature treatment in this study encompassed the labile period for sexual differentiation. The temperature sensitive period appears to have a timing and duration similar to those of the hormonal sensitive period in *O. niloticus* (Baroiller et al., 1995a,b), *M. menidia* (Conover and Fleisher, 1986), *O. bonairensis* (Strussman et al., 1997) and Japanese flounder, *Paralichthys olivaceus* (Kitano et al., 1999). Although we used a shorter period for the DES treatment (11 days) than for the heat treatment period (21 days), similar feminization rates were obtained. Baroiller and D'Cotta (2001) stated that a short temperature treatment (10 days) can be just as efficient as a longer (21 days) hormonal treatment since both cover the same critical period (14-24 days after fertilization). Wang and Tsai (2000) showed that a high temperature (32ºC) applied from 10 days post-hatching produced a high proportion of males in *O. mossambicus*. Therefore, the hypothesis that the temperature sensitive and hormonal sensitive periods have a similar timing and duration should not be discounted.

The coinciding temperature and hormonal sensitive periods might result from the effect of high temperature on the action of a hormone or enzyme during sex differentiation (Hunter et al., 1982). A temperature effect on the steroidogenic enzyme genes that control the production of aromatase (the catalyst for the breakdown of androgens to estrogen) was reported by Pieau et al. (1994), Wibbels et al. (1994) and Crews (1996) in thermosensitive reptiles. *O. niloticus* XX females, masculinized by heat treatment, and genetic males treated at 35ºC had lower levels of aromatase gene expression (D'Cotta et al., 2001) as did *P. olivaceus* (Kitano et al., 1999). Strong aromatase activity was found only in females at a normal temperature (18°C) while aromatase was suppressed in a male group produced by heat treatment at 27ºC. In *O. niloticus,* Kitano et al. (1999) reported that high temperature (36ºC) caused significant feminization (35.5%) while a dietary aromatase inhibitor suppressed the feminization (1.1% female) in YY males. High temperature did not significantly affect the sex ratio in XY males but it significantly masculinized XX females (62.5%) and oral administration of aromatase inhibitor resulted in 100.0% and 99.0% males at either 36ºC or 28ºC, respectively. The authors proposed that low aromatase activity during the crucial period always results in masculinization, regardless of temperature or genotype, inferring that expression of the ovarian aromatase gene

was down-regulated at high temperatures in genetic females and up-regulated at the same temperature in genetic males. These studies suggest that temperature controls the aromatase activity or transcription factors.

A significantly higher number of intersex progeny were obtained in the heat treatment than in the DES treatment. Intersex fish occur in fish exposed to hormone treatment, sewage and industrial effluents (Rolland, 2000). The occurrence of intersex progeny in fish treated by hormones may indicate incomplete sex reversal and was reported by Rothbard et al. (1981) and Mair et al. (1987). However, intersex fish seem to be rare or absent in heat manipulation experiments. Mair et al. (1990) reported 9.34%, 0.1% and 4.9% intersex progeny in one cross of *O. aureus*, *O. niloticus* and *O. mossambicus*, respectively, reared at a low temperature (20ºC). No intersex fish were observed in a high temperature treatment of YY males (Abucay et al., 1999) or putative all-female progenies of *O. niloticus* (Baroiller, 1995a,b). Masculinization (86%) was obtained in a high temperature treatment of XX female progeny (D'Cotta et al., 2001), however, some individuals had an intersex feature, i.e., one normal male gonad and one sterile gonad (20.0%), male gonads with areas devoid of germ cells (54.3%) or gonads containing oocytes dispersed among lobules (11.4%). Since the occurrence of intersex individuals is common in progeny treated with a suboptimal dose or duration in hormone treatments, the appearance of intersex individuals in the high temperature treatment may indicate that the 21-day duration in our study is insufficient to feminize YY male *O. niloticus.* On the other hand, even though a longer heat treatment period (30 days at 36ºC) was used by Kwon et al. (2002), 13.0% intersex individuals were produced. They suggested that YY fish have a higher androgen:estrogen ratio than XY fish and can produce even higher androgen levels at 36ºC. Such an androgen level might trigger aromatase gene expression in differentiating gonads, resulting in 'paradoxical feminization' (complete or partial sex change), since when an aromatase inhibitor was used, no intersex individuals were produced (Kwon et al., 2002).

The successful feminization of the YY genotype is vital in producing large numbers of YY male for use as broodstock in commercial production of all-male tilapia. Males grow better than females and all-male production is the most effective solution to the problems of early sexual maturity, uncontrolled reproduction and stunted growth. From the present study, it can be concluded that high temperature treatment, which is environmentally more friendly and less harmful to human health than hormones, can be used for sex-reversal of YY all-male progenies of *O. niloticus*. However, the low survival rate and high number of intersex individuals may limit the commercial use of this technique. Therefore, the duration of the high temperature treatment may need to be optimized by, for example, using stress reducing dietary supplements. Harpaz et al. (1999) reported that dietary L-carnitine supplementation significantly improved survival rates in the ornamental cichlid, *Pelvicachromis pulcher*, following exposure to a cold shock. Even though low numbers of YY neofemales could be produced, large numbers of YY male broodstock could be generated from YY neofemale x YY male crosses without the need for time-consuming progeny testing. Treatment by high temperature can successfully be used in countries where hormone use is illegal and/or consumer reaction to hormonally-treated fish is negative. The mechanism causing intersex individuals needs to be investigated.

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